Serum neutrophil gelatinase B-associated lipocalin and matrix metalloproteinase-9 (NGAL-MMP-9) complex as a surrogate marker for mucosal healing in patients with Crohn's disease.

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SHORT TITLE: NGAL-MMP-9 complex as a surrogate marker for CD

ABBREVIATIONS: Neutrophil gelatinase B-associated lipocalin (NGAL), matrix metalloproteinase-9 (MMP-9), complete healing (CH), partial healing (PH), no healing (NH), histological healing (HH).

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ABSTRACT

Background and aims. Although costly and uncomfortable for the patient, the current standard to assess mucosal healing in Crohn's disease (CD) patients is endoscopy. The aim of this study was to evaluate NGAL-MMP-9 as surrogate marker for mucosal healing in CD patients.

Methods. Serum NGAL-MMP-9 levels were determined with sandwich ELISA before and up to 5 years after first infliximab infusion in 108 active CD patients (median age at first infliximab 36 years, 57% female) and 43 healthy controls (HC, median age 27 years, 60% female). Serum samples were matched to the time of endoscopy and complete endoscopic healing was defined as absence of ulcerations. Histological healing was defined as absence of epithelial damage (D'Haens score).

Results. At baseline, median [IQR] NGAL-MMP-9 levels were significantly higher in active CD patients *versus* HC (77.6 [36.9-141.0] *vs* 25.5 [17.8-42.8] ng/ml; $p<0.001$). After treatment, NGAL-MMP-9 levels significantly decreased in completely healed CD patients (n=38) (84.5 [36.7-138.4] to 23.4 [7.4-42.5] ng/ml; $p<0.001$) and - to a lesser extent - in non-healed CD patients (n=36) (100.9 [43.4-152.6] to 43.8 [27.0-96.8] ng/ml; $p=0.001$). ROC analysis defined a NGAL-MMP-9 cut-off level of 45 ng/ml corresponding to complete endoscopic healing (AUC=0.79, 82% sensitivity, 65% specificity) and histological healing (AUC=0.72, 79% sensitivity, 53% specificity). At baseline, CRP was not elevated in 33% of active CD patients, whereas 53% of these patients did have elevated NGAL-MMP-9 levels.

Conclusions. In the search for surrogate markers to assess mucosal healing in IBD, NGAL-MMP-9 supplements and outperforms CRP in both UC and CD patients.

KEYWORDS: NGAL, MMP-9, Crohn's disease.

INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing disease of the gastro-intestinal tract with increasing prevalence and incidence in both industrialized and developing countries [1]. In CD, a defective acute immune response with impaired neutrophil accumulation and interleukin (IL)-8 production is observed [2,3]. This may lead to delayed or incomplete removal of bacteria that breach the mucosal barrier. These bacteria will subsequently be taken up by macrophages [4] that elicit a granulomatous reaction [5] and produce a secondary chronic inflammation [3]. However, neutrophil migration to the active site of inflammation is stimulated by bacterial components e.g. through the pro-inflammatory NOD2/CARD15 pathway.

The primary function of neutrophils is to kill bacteria with the use of potent digestive enzymes present in and secreted from the granules. Neutrophil gelatinase B-associated lipocalin (NGAL, lipocalin-2) [6,7] is found in secondary neutrophil granules. NGAL is expressed in response to Toll-like receptor activation during infections [8,9] and can inhibit bacterial growth by sequestering iron-laden siderophores [10]. Moreover, NGAL has been correlated with parameters of active disease in IBD patients [11-16]. Tertiary neutrophil granules contain matrix metalloproteinase-9 (MMP-9, gelatinase B), a member of the MMP family [17]. MMPs are zinc-dependent endopeptidases involved in many developmental processes, including angiogenesis, wound healing and extracellular matrix (ECM) degradation. Dysregulated MMP-9 levels have been previously described in IBD [11,18-26]. Recently, the EMBARK study showed that a combination of fecal calprotectin, serum MMP-9 and serum IL-22 had a strong association with imaging/endoscopy-defined inflammation [27]. Moreover, neutralizing antibodies with tissue inhibitor of MMPs (TIMP)-like mechanisms against MMP-2 and MMP-9 were shown to

attenuate the development of colitis in IBD mouse models [28]. In addition to their separate circulating forms, MMP-9 and NGAL occur in covalent complexes, mainly in neutrophil degranulates [17]. However, the functional role of this NGAL-MMP-9 complex is still debated. One hypothesis is that by formation of this complex, NGAL protects MMP-9 from autodegradation [29].

In few studies NGAL or MMP-9 levels were investigated separately in blood or biopsies after anti-inflammatory treatment in patients with CD [30,31]. Based on our original view that by measuring the covalent complex of NGAL with MMP-9 we would evaluate two markers in one assay and eventually combine the information content of two assays, we recently described that serum NGAL-MMP-9 complex levels decrease after infliximab treatment in UC patients achieving mucosal healing and that levels correlate well with the Mayo endoscopic subscore [32]. With the present study, we aimed to investigate whether NGAL-MMP-9 complex levels are elevated in CD patients in comparison to healthy controls and whether levels decrease after anti-inflammatory treatment in patients with mucosal healing. Moreover, since CRP is not elevated in 20-30% of CD patients [33], we aimed to compare the diagnostic accuracy of NGAL-MMP-9 with CRP.

MATERIALS AND METHODS

Patient sampling.

Consecutive serum sampling and endoscopy were performed in 108 CD patients before and after first treatment with infliximab (Remicade; Centocor). Baseline characteristics of the CD patients are shown in **Table 1**. The baseline samples were obtained from active CD patients within a month before first infusion of infliximab and follow-up samples up to five years after start of treatment. The median (IQR) time to follow-up endoscopy was 13 (6-54) weeks. The median (IQR) interval between follow-up serum sampling and last infusion with infliximab was 49 (30-57) days. Serum samples were matched to a nearby endoscopy with a maximum interval of 30 days between serum sampling and time of endoscopy. CRP levels and neutrophil counts were measured in a centralized laboratory facility before and after infliximab, at time points corresponding to endoscopy. Furthermore, we collected serum samples from 43 healthy (non-IBD) controls (HC, median age 27 years, 60% female). From all individuals a written informed consent was obtained and the study was approved by the University Hospital Ethics Committee (*Vlaams erfelijkheidsonderzoek Crohn en colitis ulcerosa* (VLECC) S-53684).

Definition of endoscopic and histological healing.

Endoscopic mucosal healing was determined by expert gastroenterologists (SV, MF, PR and GVA) at follow-up endoscopic evaluation. Complete endoscopic healing was defined as absence of ulcerations, whereas partial healing was defined as significant endoscopic improvement, but with ulcerations still present. Histopathological analysis was performed on haematoxylin and eosin (H&E) stained mucosal biopsies. The pathologist (GDH) was blinded to the patient ID, disease status and treatment. The slides were scored using the D'Haens histological scoring

system [34] which is comprised of 8 subcategories mounting to a maximum total score of 16 (also represented in **supplementary table 1**). Histological healing was defined as an absence of epithelial damage.

Sandwich ELISA.

The commercial anti-human NGAL-MMP-9 complex ELISA kit (R&D Systems, Abingdon, UK) was used to determine NGAL-MMP-9 complex levels in the serum of CD patients and HC according to the manufacturer's guidelines. Briefly, two antibodies with different antigen specificities were used: an antibody directed towards MMP-9 was pre-coated on the plate and another against NGAL was used as the detection antibody. Hence, only NGAL-MMP-9 complexes are measured. The absorbance was measured at 450nm with a spectrophotometer (Omega, Nazareth, Belgium). NGAL-MMP-9 complex levels were quantified with the use of a calibration curve using purified human NGAL-MMP-9 as a standard (Mars software, BMG labtech, Ortenberg, Germany).

Statistical analysis.

Data were analyzed with SPSS Statistics 20.0 software (SPSS Inc., Chicago, IL) with the use of the non-parametric Mann-Whitney *U*-test for unpaired samples and Wilcoxon signed-rank test for paired samples. Spearman correlation analysis (correlation coefficient=r), Kendall's tau rank correlation (correlation coefficient=τ), Fisher's exact test, Chi Square test, receiver operating characteristic (ROC) analysis and binary logistic regression analysis were also performed in SPSS. P-values of <0.05 were considered significant.

RESULTS

Serum NGAL-MMP-9 complex levels are increased in active CD patients and decrease after treatment with infliximab.

Complete mucosal healing with absence of ulcerations at follow-up endoscopy was seen in 38 CD patients (35%) after treatment. Thirty-four patients (32%) presented clear endoscopic improvement, but some ulcerations could still be observed. In contrast, 36 CD patients (33%) did not show any signs of endoscopic improvement or presented even worsened lesions. Serum NGAL-MMP-9 complex levels were elevated at baseline in active CD patients as compared to HC ($p<0.001$) (**Figure 1A and Table 2**). After treatment, NGAL-MMP-9 levels significantly decreased in completely healed CD patients ($p<0.001$), although 4 patients (10%) showed increased NGAL-MMP-9 levels after therapy **(Figure 1A and Table 2)**. Patients with partial healing had mild decrease of NGAL-MMP-9 levels after treatment ($p=0.048$) (**Figure 1A and Table 2**) and the decrease of NGAL-MMP-9 levels (difference between before and after treatment) was significantly lower than in patients with complete healing ($p=0.001$). Moreover, 10 patients with partial healing (34%) had increased levels after treatment. In non-healed CD patients, NGAL-MMP-9 serum levels also decreased after treatment ($p=0.001$) **(Figure 1A and Table 2)**. However, the decrease was significantly less profound than in completely healed CD patients ($p=0.020$). No significant difference in extent of decrease of NGAL-MMP-9 levels was observed in completely healed patients compared to partially healed patients ($p=0.294$). Moreover, NGAL-MMP-9 levels in completely healed CD patients decreased after treatment to levels equivalent to HC levels (median [IQR] 23.4 [7.4-42.5] *versus* 25.5 [17.8-42.8], $p=0.131$), whereas NGAL-MMP-9 levels in partially healed or non-healed CD patients remained elevated

after treatment in comparison to HC levels (median [IQR] 43.5 [18.7-61.8] and 43.8 [27.0-96.8] *versus* 25.5 [17.8-42.8]; p=0.041 and p=0.016, respectively) **(Figure 1A and Table 2)**. No significant differences were found between NGAL-MMP-9 levels in complete, partial or non-healed CD patients at start of treatment **(Figure 1A and Table 2)**.

Finally, we investigated whether patients at time of follow-up endoscopy maintained under the same type of concomitant treatment as at the time of first infliximab infusion. We found that 56%, 83%, 96% and 73% of the patients who received 5-ASA, AZA/6-MP, MTX or corticosteroids, respectively, maintained on the same concomitant treatment at follow-up endoscopy. A proportion of patients stopped 5-ASA or corticosteroid treatment. However, we recorded no influence of the type of concomitant treatment at follow-up endoscopy on the outcome of mucosal healing (**Supplementary table 2**).

Serum NGAL-MMP-9 complex levels correlate with neutrophil counts and complement CRP as an inflammatory marker.

To assess its role as a serum marker of inflammation and mucosal healing, we correlated serum NGAL-MMP-9 complex levels with neutrophil counts and CRP levels. NGAL-MMP-9 serum levels correlated well with the amount of neutrophils in the blood (Spearman's Rho [r] = 0.470, p<0.001). For most of the patients, neutrophil counts were found to be in the clinically determined interval of 2.5–7.8 10^9/L. Nevertheless, 25% of the CD patients presented with neutrophilia (>7.8 10^9/L). Neutrophil counts significantly decreased after treatment in completely healed CD patients (p<0.001) **(Table 2** and **Figure 1C)**, whereby 13% of the patients showed neutropenia with neutrophil counts lower than 2.5 10^9/L. CD patients with partial healing after

treatment also showed a decrease in neutrophil counts (p=0.002) **(Table 2** and **Figure 1C)**. However, this decrease had a trend to be less profound than in patients with complete healing (p=0.118). In CD patients without healing, neutrophil counts also decreased after treatment (p=0.005), but to a lesser extent than in patients with complete healing (p=0.153) **(Table 2** and **Figure 1C)**. The decrease of neutrophil counts was not significantly different between partially healed or non-healed CD patients (p=0.928). Furthermore, no significant difference was observed between baseline neutrophil counts of CD patients with complete healing compared to CD patients without mucosal healing (p=0.95) **(Table 2** and **Figure 1C)**. The amount of neutrophils correlated with CRP levels (r=0.357, p<0.001). In patients with high CRP levels, the neutrophil count was also elevated (>5.4 10^9/L) in 56% of the patients, whereas in patients with low CRP levels 70% of the patients also had low neutrophil levels. In case of low or high NGAL-MMP-9 levels, neutrophil counts were high (>5.4 10^9/L) in 58% of patients with high NGAL-MMP-9 (>45 ng/ml) and in 73% of the patients neutrophils counts were low (<5.4 10^9/L) when NGAL-MMP-9 was also low (<45 ng/ml). Furthermore, we calculated the ratio of NGAL-MMP-9 levels over the amount of neutrophils (ng/ml/10^9/L) to investigate whether the decrease of NGAL-MMP 9 levels only reflected the decrease in number of neutrophils. With ROC analysis, an AUC of 0.74 could be determined for the ratio to discriminate complete mucosal healing (**Supplementary figure 1**), which was lower than the AUC of NGAL-MMP-9 as such (AUC=0.78).

A good correlation was detected between NGAL-MMP-9 and CRP levels (r=0.448, p<0.001). In 67% of active CD patients, CRP levels were elevated (>5 mg/L) at start of treatment with infliximab. After treatment, CRP levels significantly decreased in CD patients with complete and partial mucosal healing (p<0.001 and p<0.001, respectively) (**Table 2** and **Figure 1B**). We

observed no significant difference in the decrease of CRP levels between complete and partially healed patients (p=0.420). CRP levels also decreased in CD patients without mucosal healing after treatment (p=0.037) (**Table 2** and **Figure 1B**). However, the decrease of CRP levels was more profound in complete or partial CD healers after treatment than in CD non-healers (p=0.104 and p=0.003, respectively). Of importance, CRP was not elevated (<5mg/L) in 33% of patients with active disease at start of treatment, whereas 53% of these patients did have elevated (>45 ng/ml) NGAL-MMP-9 levels. Moreover, 47% of the patients with low CRP levels did have elevated neutrophil counts (>5.4 10^9/L). This indicates that both NGAL-MMP-9 levels and neutrophil counts can be used to supplement CRP measurements in patients who do not present with elevated CRP levels.

Serum NGAL-MMP-9 complex levels correlate with endoscopic healing and are lower in patients with ileal disease.

Serum NGAL-MMP-9 complex levels correlated with endoscopic scores indicating the degree of healing (Kendall's tau [τ] = 0.296, p<0.001) as was defined at the time of endoscopic evaluation. The highest NGAL-MMP-9 complex levels were observed in patients who did not reach mucosal healing. After therapy with infliximab, NGAL-MMP-9 levels decreased rapidly and correlated with restoration of mucosal integrity. An analogue analysis was performed identifying the correlation of CRP levels (τ=0.307, p<0.001) and neutrophil counts (τ=0.239, p<0.001) with the degree of mucosal healing. NGAL-MMP-9 complex levels decreased according to the degree of mucosal healing (**Figure 2A**). In contrast, CRP was markedly elevated in CD patients who did not heal, whereas CRP levels in patients with partial and complete healing were similar and

therefore not discriminative (**Figure 2B**). Neutrophil count reflected the degree of endoscopic improvement, discriminating between no, partial and complete mucosal healing (**Figure 2C**).

Median [IQR] NGAL-MMP-9 levels were significantly lower (36.8 [10.4-69.1] ng/ml) in patients with active ileal disease (n=11) compared to patients with active (ileo)colonic disease (84.0 [40.3-147.6] ng/ml) before start of treatment (p=0.008) (**Supplementary figure 2A and B**). No significant difference was observed in NGAL-MMP-9 levels at start of treatment of completely (n=6) or partially (n=3) healed CD patients with ileal disease compared with non-healed (n=2) CD patients with ileal disease (p=0.286 and p=0.200, respectively) (**Supplementary figure 2B**). Since neutrophils are the source of NGAL-MMP-9, we further compared the amount of neutrophils in patients with ileal disease compared to (ileo)colonic disease. However, the difference was not significant (p=0.069) with a median (IQR) neutrophil count of 5.0 (2.9 – 6.9) 10^9/L in patients with ileal disease and 6.0 (4.3-7.9) 10^9/L in patients with (ileo)colonic disease.

Serum NGAL-MMP-9 complex can discriminate complete mucosal healing as defined by endoscopic evaluation.

ROC analysis was performed to evaluate the performance of NGAL-MMP-9, CRP and neutrophil levels to discriminate mucosal healing. When including patients with partial mucosal healing into the non-healing group of patients, the area under the curve (AUC) for NGAL-MMP-9 levels was 0.77 and levels lower than 45 ng/ml were determined to discriminate complete mucosal healing with 82% sensitivity, 60% specificity, 29% positive predictive value (PPV) and 95% negative predictive value (NPV). The AUC for CRP levels was 0.74 and levels lower than 5

mg/L were able to discriminate complete mucosal healing with 79% sensitivity, 57% specificity, 28% PPV and 93% NPV. Neutrophil levels lower than 5.4 10^9/L were able to discriminate complete mucosal healing with an AUC of 0.68, sensitivity of 79%, specificity of 48%, 25% PPV and 91% NPV.

ROC analysis whereby patients with partial mucosal healing were excluded indicated that NGAL-MMP-9 complex levels lower than 45 ng/ml could discriminate complete mucosal healing from no healing with a sensitivity of 82% and specificity of 64% (**Figure 3A and Table 3**). The AUC was 0.79 and a PPV of 44% and NPV of 91% were determined. The diagnostic accuracy of NGAL-MMP-9 was 68%. ROC analysis with CRP levels showed a comparable AUC of 0.75. CRP levels lower than 5 mg/L were able to discriminate complete mucosal healing with 79% sensitivity, 58% specificity, 39% PPV and 89% NPV (**Figure 3A and Table 3**). To investigate the superiority of NGAL-MMP-9 over CRP, we analyzed the performance of NGAL-MMP-9 to discriminate complete mucosal healing in patients with low CRP levels at baseline. Of the 36 patients with low CRP, 18 patients had complete healing and 11 patients had no healing after treatment. An AUC of 0.78 was determined and a cut-off value of 38 ng/ml NGAL-MMP-9 was able to discriminate complete mucosal healing with 83% sensitivity, 58% specificity, 47% PPV and 88% NPV. Finally, a neutrophil count lower than 5.4 10^9/L was able to discriminate complete mucosal healing with an AUC of 0.70, 79% sensitivity, 52% specificity, 36% PPV and 88% NPV (**Figure 3A and Table 3**).

In order to investigate whether the combination of NGAL-MMP-9 and CRP in clinical practice would improve the prediction of complete mucosal healing, we performed binary logistic regression analysis including both parameters. With the use of the predicted probabilities, ROC

analysis was performed and indicated that the combination of the two markers was able to discriminate complete mucosal healing with an AUC of 0.81, 82% sensitivity, 73% specificity, 51% PPV and 92% NPV (**Figure 3A and Table 3**). The combination of three markers (NGAL-MMP-9, CRP and neutrophils) did not improve the discriminative power (AUC of 0.81) as compared with the combination of two markers (NGAL-MMP-9 and CRP).

Finally, we evaluated the diagnostic value of NGAL-MMP-9 to discriminate between UC and CD patients. In our previous study [32], we identified a median (IQR) NGAL-MMP-9 level of 87.3 (43.2-161.9) ng/ml in active UC patients before start of treatment. In the present CD cohort, the median (IQR) NGAL-MMP-9 level was 77.6 (36.9-141.0) ng/ml in active patients at start of treatment with infliximab. Based on these data, we could not document a significant difference between active UC and CD patients (p=0.268) (**Supplementary figure 3 with means ± SEM**).

Serum NGAL-MMP-9 complex levels correlate well with histological activity and can discriminate histological healing.

Histological activity was determined in a subset of CD patients (n=70) with the use of the D'Haens score. A good correlation was determined between endoscopic and histological activity scores (r=0.656, p<0.001). Twenty-eight CD patients showed histological healing with absence of epithelial damage after treatment of whom 21 patients (75%) also had complete mucosal healing based on endoscopic evaluation and 7 (25%) had partial mucosal healing. A high concordance was found especially in patients with active disease, since all 19 patients with no endoscopic healing also had no histological healing. Of the patients with partial endoscopic healing, however, only 32% (7 out of 22) had histological healing. Importantly, 21 out of 29

patients (72%) with complete endoscopic healing also had histological healing with an absence of epithelial damage.

NGAL-MMP-9 levels correlated well with histological healing (τ =0.238, p=0.001) (**Figure 2D**), whereas CRP levels and neutrophil count had lower correlation factors (τ=0.197, p=0.006 and τ=0.118, p=0.099; respectively) (**Figure 2E-F**). Intriguingly, NGAL-MMP-9 levels and neutrophil counts were lower in patients with ileal disease (*vide supra*). Histopathological investigation showed that all patients with ileal disease (n=8) had moderate infiltration of polymorphonuclear (PMN) cells in the lamina propria and no crypt abscesses were seen in the epithelium. On the contrary, 15% of patients with (ileo)colonic disease did have severe increase of PMN cells in the lamina propria, 40% of the patients had cryptitis and 22% presented with crypt abscesses in the epithelium.

With ROC analysis, we identified that NGAL-MMP-9 levels lower than 45 ng/ml could discriminate histological healing with an AUC of 0.72, 79% sensitivity, 53% specificity, 29% PPV and 91% NPV (**Figure 3B and Table 3**). In contrast, CRP levels lower than 5 mg/L were not as potent as NGAL-MMP-9 to discriminate histological healing (AUC=0.68, 75% sensitivity, 54% specificity, 29% PPV and 90% NPV) (**Figure 3B and Table 3**). A neutrophil count lower than 5.4 10^9/L was the least potent marker (AUC=0.60, 68% sensitivity, 46% specificity, 24% PPV and 85% NPV) (**Figure 3B and Table 3**). Finally, the combination of NGAL-MMP-9 and CRP was not better than NGAL-MMP-9 alone to discriminate histological healing (AUC=0.72, 54% sensitivity, 79% specificity, 39% PPV and 87% NPV) (**Figure 3B and Table 3**).

DISCUSSION

Identification of non-invasive biomarkers has become an important research topic since frequent endoscopic examinations are costly and uncomfortable for the patient. Several serological and fecal markers have been investigated for their use in diagnosis and assessment of disease activity, but none have been shown to be superior to the commonly used CRP [35]. In this study, we investigated the diagnostic accuracy of NGAL-MMP-9, CRP and neutrophil levels in a considerable CD cohort and found that NGAL-MMP-9 performed better than CRP and neutrophil count in discriminating complete endoscopic and histological healing. With ROC analysis, we showed that NGAL-MMP-9 was the best discriminator of complete endoscopic healing and that the combination with CRP discretely enhanced the discriminative power. Importantly, we found that CRP was not elevated in one third of CD patients with active disease at start of treatment, whereas more than half of these patients did have elevated NGAL-MMP-9 levels. This was further investigated by ROC analysis, identifying that NGAL-MMP-9 was a good surrogate marker to discriminate complete mucosal healing in patients without elevated CRP levels at baseline. These data suggest that NGAL-MMP-9, which is a combination of two marker molecules in one assay, can be considered as a new surrogate marker for IBD patients to assess mucosal healing and can complement or even replace CRP measurements. In a previous study, we already showed the value of the NGAL-MMP-9 complex as surrogate marker for mucosal healing in UC patients [32].

Besides CRP, other markers currently used include fecal calprotectin. Fecal calprotectin is a sensitive marker of intestinal inflammation and correlates well with the degree of endoscopic activity [36]. Patients with ileal CD are reported to have significantly lower fecal calprotectin levels

than those with (ileo)colonic disease even in the presence of large and/or very large ulcers [37]. Moreover, the time of sampling during the day may affect calprotectin levels [38]. In our cohort, we found that NGAL-MMP-9 levels were lower in patients with active ileal disease compared to patients with (ileo)colonic disease before start of treatment (**Supplementary figure 2A and B**). Since neutrophils are the source of both calprotectin and NGAL-MMP-9, we further looked whether the amount of neutrophils was lower in patients with ileal disease compared to (ileo)colonic disease, however, we could not document a significant difference.

A limitation of our study is the lack of fecal calprotectin measurements for a direct comparison with NGAL-MMP-9. Interestingly, the EMBARK study showed that a combination of fecal calprotectin, serum MMP-9 and serum IL-22 had a strong association with imaging/endoscopy-defined inflammation [27]. Moreover, recent reports indicate the emergence of fecal MMP-9 levels in the IBD biomarker field. Annahazi *et al.* described that fecal MMP-9 is a good marker for the non-invasive evaluation of disease activity and mucosal healing in UC [39]. Moreover, Kolho *et al.* showed that fecal MMP-9 performed equally well as fecal calprotectin in UC, suggesting its use as a surrogate marker of inflammation [19]. Recently, fecal MMP-9 was also tested in a cohort of CD patients, but was not correlated with any of the activity indices of CD [40]. Therefore, it might be interesting, in the future, to evaluate NGAL-MMP-9 complex levels in fecal samples.

As was shown by previous studies, endoscopic mucosal healing does not necessarily reflect quiescent histological disease [41,42]. Most of the studies investigating histological healing were performed in UC patients and there is scarce information in CD [43]. In the present study, we used the D'Haens score [34], which is also known in literature as the Colonic or Ileal Global Histologic Disease Activity Score (GHAS). Despite the evidential importance of microscopic activity,

histological remission has yet to be recommended as a therapeutic endpoint for clinical trials or practice in IBD [44]. In our cohort of CD patients, we found a good correlation between histological and endoscopic mucosal healing. A high concordance was found especially in patients with active disease. Importantly, 72% of the patients with complete endoscopic healing also had histological healing with an absence of epithelial damage. These data are in line with current literature since it was reported that persistent histological inflammation occurs in 25–37% of patients with clinical and endoscopic quiescent CD [45,46].

Since CD is characterized by transmural disease, endoscopic and histological mucosal evaluations are not able to actually determine "deep" remission in CD patients. Recent studies indicate that magnetic resonance enterography (MRE) can evaluate ulcer healing with a high level of accuracy compared to ileocolonoscopy [47]. This method is, however, not standardly performed in all patients, is costly and requires technical and analytical skills. Moreover, it has been recommended that the combination of mucosal and histological healing should be achieved as a minimum therapeutic target in IBD patients [48].

In order to investigate the diagnostic value of NGAL-MMP-9, we compared the levels between active UC and CD patients; however, we could not document a significant difference **(Supplementary figure 3)**. Moreover, with ROC analysis we defined a higher cut-off value (97.7 ng/ml) in our UC cohort than in the present CD cohort (45 ng/ml). This may be in part due to the fact that, in general, higher NGAL-MMP-9 levels were found in UC patients compared to CD patients. In addition, the cut-off values were chosen in order to identify as accurate as possible patients with mucosal healing. In the UC cohort, a high specificity of the test was chosen since levels higher than 97.7 were positively associated with no healing. In the present

CD cohort, a high sensitivity was chosen, since levels lower than 45 ng/ml were positively associated with complete mucosal healing.

Although NGAL and MMP-9 have been discussed separately as good markers of disease [13-16,27], our previous study in UC patients was the first to investigate the NGAL-MMP-9 complex as a surrogate serum marker of mucosal healing [32]. Urinary NGAL-MMP-9 has been reported to predict pediatric IBD [11] and in cancer research NGAL-MMP-9 complex is a well-known biomarker [49-51].

In conclusion, we propose that serum NGAL-MMP-9 complex is useful and recommended as a surrogate marker of endoscopic and histological mucosal healing after treatment with infliximab in both UC and CD patients. NGAL-MMP-9 complex hereby outperforms CRP and can be used as a single marker in patients without elevated CRP levels or in combination with CRP to discriminate mucosal healing. Prospective studies to evaluate this new marker are needed and the efficacy of the marker to discriminate mucosal healing under other emerging gut-selective biological treatments (e.g. vedolizumab) should be investigated.

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563 **FIGURES**

564 **Figure 1**: Serum NGAL-MMP-9 complex (**A**), CRP (**B**) and blood neutrophil (**C**) levels before
565 and after therapy with infliximab in CD patients with complete (CH), partial (PH) and no healing
566 (NH). Healthy control levels of NGAL-MMP-9 are also depicted. The dotted lines represent the
567 clinically used cut-off value of 5 mg/L CRP and the neutrophil count interval of 2.5 - 7.8 10^9/L.
568 P-values <0.001, <0.01 and <0.05 are depicted as ***, ** and * respectively. P-values >0.05 are
569 indicated as NS (non-significant). Paired data were analyzed using the Wilcoxon signed rank
570 test, whereas unpaired data were analyzed using the Mann Whitney-*U* test.

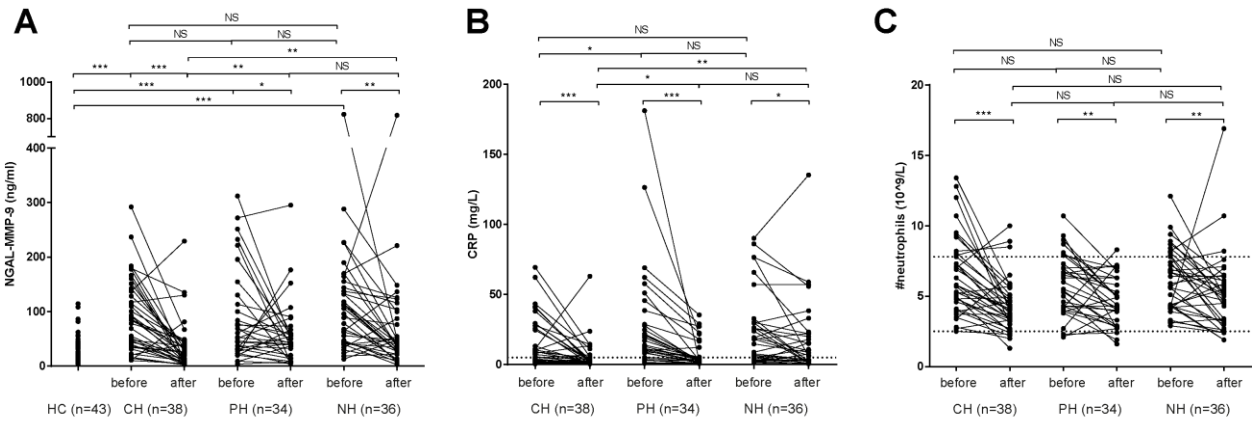

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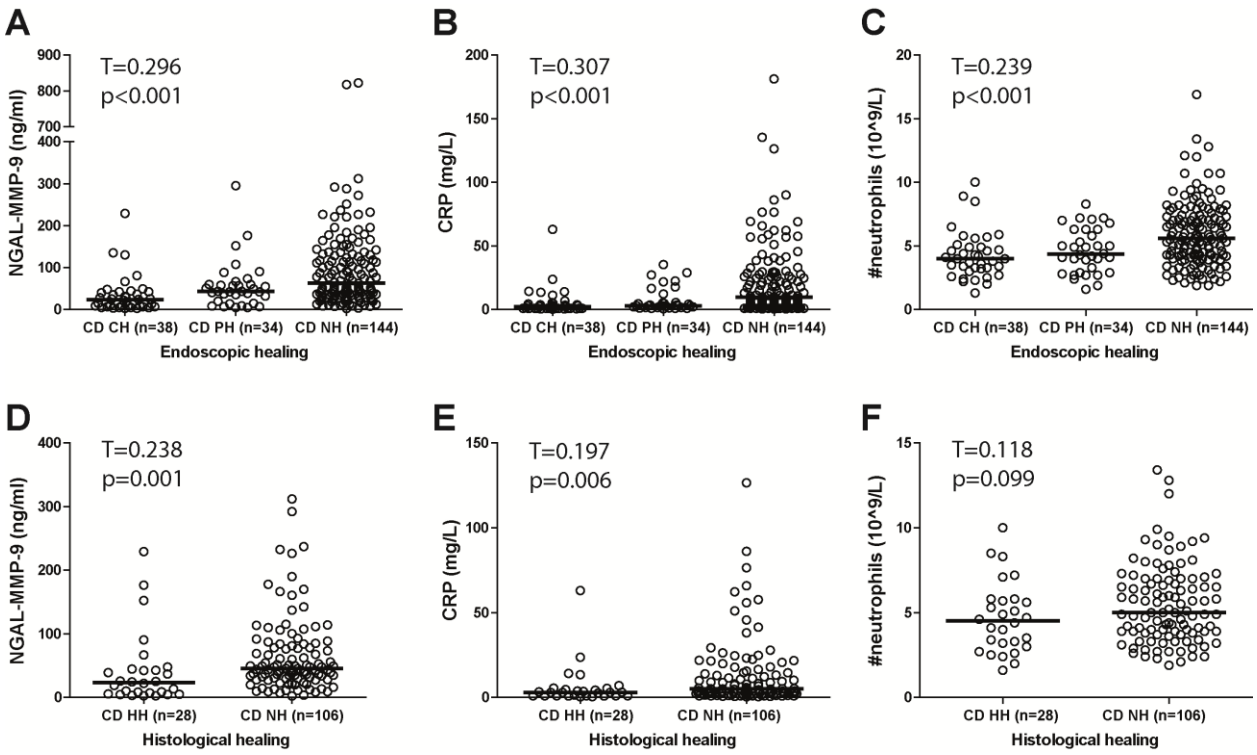
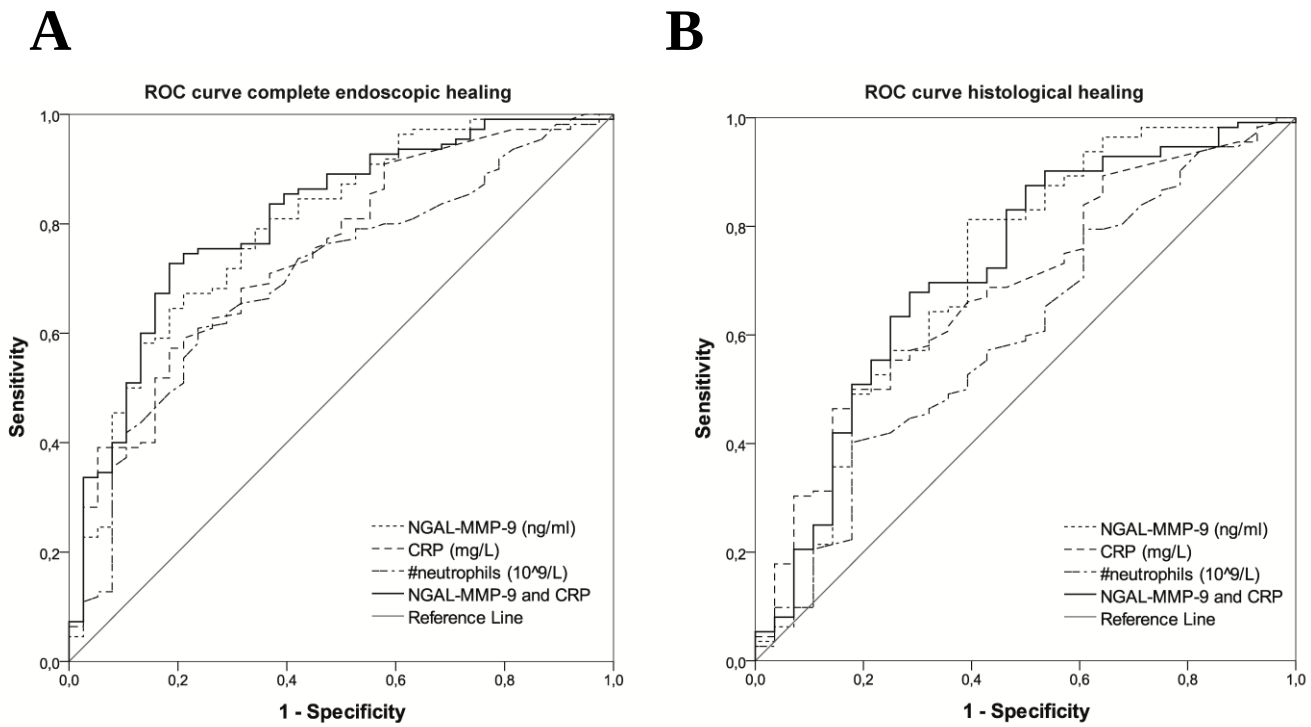
Figure 2: Distributions of NGAL-MMP-9 complex levels, CRP levels and neutrophil counts with endoscopic and histological healing scores. The top panels illustrate the levels of NGAL-MMP-9 (**A**), CRP (**B**) and neutrophils (**C**) with the corresponding endoscopic healing scores, regardless of their response to therapy or time point before or after treatment. In the lower panels, the levels of NGAL-MMP-9 (**D**), CRP (**E**) and neutrophils (**F**) were grouped according to the corresponding histological healing scores. Abbreviations: CH, complete healing; HH, histological healing; PH, partial healing; NH, no healing; T, Kendall's tau correlation factor.



584 **Figure 3: ROC analysis of serum NGAL-MMP-9 complex values, CRP levels and**
585 **neutrophil counts corresponding to complete endoscopic (A) and histological healing (B).**

586 The cut-off values, AUC, specificity, sensitivity, PPV and NPV of NGAL-MMP-9, CRP,
587 neutrophil count and the combination of NGAL-MMP-9 with CRP to discriminate complete
588 mucosal healing and histological healing are shown in **Table 3**. The ROC curve for NGAL-
589 MMP-9 levels is depicted as a dotted line (…), for CRP levels as stripes (- - -) and neutrophil
590 count as alternating stripes and dots (- . -).

591



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TABLES

Table 1: Patient characteristics and laboratory markers at the start of first treatment with infliximab for CD patients with complete, partial and no mucosal healing.

Baseline patient characteristics	**CH (n=38)**	**PH (n=34)**	**NH (n=36)**	p-value*
Male/female (%)	14/24 (37/63)	16/18 (47/53)	16/20 (44/56)	0.66[b]
Median (IQR) age at first infliximab (years)	41.0 (30.8-50.3)	34.3 (25.8-43.6)	34.7 (23.7-45.6)	0.21[a]
Median (IQR) duration of disease prior to first infliximab (years)	7.5 (3.0-21.9)	7.6 (3.5-16.0)	11.6 (2.0-18.6)	0.93[a]
Median (IQR) CRP at first infliximab (mg/L)	6.4 (1.7-26.5)	13.5 (7.5-31.2)	16.5 (3.3-29.7)	0.09[a]
Normal CRP (<5mg/L) levels at baseline (%)	18 (47)	7 (21)	11 (31)	0.05[b]
Median (IQR) amount of neutrophils (10^9/L)	5.8 (4.5-8.0)	5.8 (4.2-7.5)	6.2 (4.3-7.9)	0.71[a]
Active smoking at first infliximab (%)	13 (38)	7 (21)	9 (27)	0.13[b]
Montreal classification				
Age at diagnosis (%)				0.48[b]
A1 (<16 years)	4 (11)	5 (15)	5 (14)	
A2 (17-40 years)	27 (71)	26 (76)	29 (81)	
A3 (>40 years)	7 (18)	3 (9)	2 (5)	
Location of disease (%)				0.53[b]
L1 (ileum)	6 (16)	3 (8)	2 (6)	
L2 (colon)	9 (24)	6 (18)	10 (27)	
L3 (ileocolon)	23 (60)	25 (74)	24 (67)	
L4 (upper GI)	1 (3)	0 (0)	2 (6)	
Disease behavior (%)				0.77[b]
B1 (non-stricturing, non-penetrating)	12 (32)	7 (21)	11 (31)	
B1p	8 (21)	4 (11)	6 (17)	
B2 (stricturing)	8 (21)	8 (24)	5 (14)	
B2p	5 (13)	8 (24)	9 (25)	
B3 (penetrating)	3 (8)	3 (9)	1 (3)	
B3p	2 (5)	4 (11)	4 (10)	
Major abdominal surgery prior to first infliximab (%)	17 (45)	18 (53)	11 (31)	0.16[b]
Concomitant medication at first infliximab (%)				
5-aminosalicylates (5-ASA)	23 (61)	17 (50)	18 (50)	0.58[b]
Corticosteroids	12 (32)	9 (26)	10 (28)	0.88[b]
Immunomodulators (AZA or MTX)	24 (63)	20 (59)	22 (61)	0.93[b]

597 **Abbreviations**: AZA, azathioprine; CH, complete healer; CRP, C-reactive protein; IQR, interquartile
598 range; MTX, methotrexate; NH, no healer; p, perianal disease modifier; PH, partial healer.
599 *$p<0.05$ was considered significant and the statistical difference was analyzed by [a] Kruskal-Wallis test or
600 [b] Chi Square test
601

602 **Table 2:** Overview of NGAL-MMP-9, CRP and neutrophil counts in CD patients before and
603 after treatment with infliximab.

		NGAL-MMP-9 (ng/ml)	**CRP (mg/L)**	**#neutrophils (10^9/L)**
HC (n=43) median (IQR)		25.5 (17.8-42.8)	NA	NA
CH (n=38) median (IQR)	Before IFX	84.5 (36.7-138.4)	6.4 (1.7-26.5)	5.8 (4.5-8.0)
	After IFX	23.4 (7.4-42.5)	2.3 (1.0-4.6)	4.0 (3.2-5.0)
	*p-value**	*<0.001*	*<0.001*	*<0.001*
PH (n=34) median (IQR)	Before IFX	57.0 (30.0-136.5)	13.5 (7.5-31.2)	5.8 (4.2-7.5)
	After IFX	43.5 (18.7-61.8)	3.0 (1.8-7.2)	4.4 (2.9-6.3)
	*p-value**	*0.048*	*<0.001*	*0.002*
NH (n=36) median (IQR)	Before IFX	100.9 (43.4-152.6)	16.5 (3.3-29.7)	6.2 (4.3-7.9)
	After IFX	43.8 (27.0-96.8)	6.3 (2.6-21.5)	5.0 (3.1-6.3)
	*p-value**	*0.001*	*0.037*	*0.005*

604 **Abbreviations**: CH, complete healer; IFX, infliximab; IQR, interquartile range; MMP-9, matrix metalloproteinase-
605 9; NA, not available; NGAL, neutrophil gelatinase B-associated lipocalin; NH, no healer; PH, partial healer.

606 *p<0.05 was considered significant and the statistical difference was analyzed by Wilcoxon signed-rank test

607

608 **Table 3:** ROC analysis specifications of serum NGAL-MMP-9 complex values, CRP levels,
609 neutrophil counts and the combination of NGAL-MMP-9 and CRP to discriminate complete
610 endoscopic and histological healing.

Serum marker	**Cut-off**	**Complete endoscopic healing**					**Histological healing**				
		AUC (95% CI)	**%sens**	**%spec**	**PPV**	**NPV**	**AUC (95% CI)**	**%sens**	**%spec**	**PPV**	**NPV**
NGAL-MMP-9	45 ng/ml	0.79 (0.71-0.88)	82	64	44	91	0.72 (0.60-0.84)	79	53	29	91
CRP	5 mg/L	0.75 (0.66-0.84)	79	58	39	89	0.68 (0.58-0.79)	75	54	29	90
#neutrophils	5.4 10^9/L	0.70 (0.61-0.80)	79	52	36	88	0.60 (0.48-0.72)	68	46	24	85
NGAL-MMP-9 + CRP	0.34 *(pred_prob)*	0.81 (0.73-0.89)	82	73	51	92	0.72 (0.61-0.83)	54	79	39	87

611 **Abbreviations**: AUC, area under the curve; sens, sensitivity; spec, specificity; PPV, positive predictive value; NPV,
612 negative predictive value; pred_prob, predicted probability as defined by binary logistic regression analysis.